Article

FACTORS AFFECTING PROLIFERATION AND DIFFERENTIATION OF LEPIDOPTERAN MIDGUT STEM CELLS

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Midgut stem cells of last instar larvae and pupae of Heliothis virescens, Lymantria dispar and several other Lepidopteran species have been cultured in vitro and have been induced to proliferate using low titers of ecdysteroids and the 77-Kda peptide fragment, α-arylphorin, isolated and identified from pupal fat body tissue. The insulin-related hormone, Bombyxin, also induced mitosis in cultured midgut stem cells; it appeared to be fast-acting and quickly inactivated, while α-arylphorin was slower to act and had a longer lasting effect in vitro, indicating different functions for these proliferation agents. Changes in Calcium ion concentration within or outside the cells discretely affected stem cell differentiation, indicating a role for second messenger participation in peptide regulation of this process. Four different peptides (MDFs 1–4) that induced midgut stem cells to differentiate to mature midgut cell types in vitro were isolated and characterized from conditioned media and hemolymph of H. virescens and L. dispar. However, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and all-trans retinoic acid (RA) from vertebrate sources induced differentiation to non-midgut cell types as well. MDF1 was located in basal areas of columnar cells of midgut epithelium, although MDF2 was observed in all of the cytoplasm of columnar cells and in droplets of antibody positive material in the midgut lumen, suggesting a digestive function as well for this

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peptide. Anti-MDF-3 stained the central areas of cultured midgut columnar cells and the bases of columnar cells of midgut epithelium in vivo. Midgut secretory cells stained with anti-MDF-4; streams of MFD-4-positive material were observed extending from secretory cells facing the epithelial lumen, and as a layer on the hemolymph-facing side, suggesting an endocrine or paracrine function for this or an immunologically similar peptide. Published 2010 Wiley Periodicals, Inc.†

INTRODUCTION

The midgut is second in size to the epidermis of lepidopteran larvae, and is the major site for food digestion, transport of nutrients, and regulation of ion balance (Billingsley and Lehane, 1996). Bacteria, viruses, and toxins may accompany ingested food and water, making the midgut an organ of interest to researchers in insect control as well as insect development.

Intermolt midgut epithelium consists of columnar and goblet cells with a smattering of endocrine and stem cells, arranged in a pattern characteristic for each species (Billingsley and Lehane, 1996). Columnar cells are believed to function primarily in digestion and transport of nutrients, while goblet cells serve as ionic regulators (Billingsley and Lehane, 1996).

Several types of endocrine cells in the midgut can be recognized by differences in morphology and their immunologic responses to a large number of peptides, including allatostatins, leukokinin (a diuretic hormone), myoinhibitory peptide, tachykinins (Veenstra et al., 2008; Veenstra, 2009), cholecystekinin, substance P, vasoactive intestinal peptide, somatoliberin, gonadoliberin (Andries and Tramu, 2004), FMRFamide, allatotropin, neuropeptide F, myosuppressins, and sulfakinins, among others (Zitnan et al., 2005) as well as to antibody raised against a differentiation factor that we isolated, sequenced, and named MDF-4 (Loeb and Jaffe, 2002).

In Lepidopteran larvae, the round stem cells, situated at the bases of columnar and goblet cells, multiply rapidly before each molt and then intercalate between existing epithelial cells as they elongate and differentiate to mature forms during the molt, maintaining the existing epidermal columnar-goblet cell pattern. Thus, a larger midgut for the next stage larva or a midgut with different digestive properties suited to pupae or adult insects results (Baldwin et al., 1996; Teffamanti et al., 2007). Stem cells can also multiply and differentiate to repair a midgut injured by insecticides, viruses, or bacterial toxins (Spies and Spence, 1985; Loeb et al., 2001) or depleted by starvation followed by feeding (Takeda et al., 2001).

As in vertebrate cultures, insect cell cultures have been known to release growth factors into their culture media. Such factors have sustained DNA and protein synthesis, mitogenesis, and/or induced development in co-cultured tissues and cell lines. Most remain undefined. Insect growth factors have also been derived from hemolymph and fat body (reviewed in Loeb et al., 1999). Testis sheaths of Lepidoptera release diffusible factors essential to the initiation of meiosis in spermatocytes (Giebultowicz et al., 1987). Insect-derived growth factor (96 KDa) has been purified and sequenced from conditioned media supporting an embryonic cell line of *Sarcophaga peregrina* (Homma et al., 1996). Allatostatins have also been identified in insect midgut tissue (Secher et al., 2001; Takeda et al., 2001), although in our hands allatostatins did not appear to affect Lepidopteran midgut stem cell multiplication or

differentiation in vitro (Loeb, unpublished data). Multi-step HPLC fractions from cockroach midgut revealed two forms of allatostatin, as well crustacean cardioactive peptide and fractions (as yet unidentified) that stimulated proliferation in cultured midgut stem cells and a fat body cell line (Takeda et al., 2001).

A few insect growth factors that resemble vertebrate growth factors have been identified. Mammalian insulin-like growth factors have induced resumption of mitosis after serum deprivation in a Lepidopteran cell line (Hatt et al., 1997), and in this review we discuss Bombyxin, an insulin family member that induces midgut stem cell proliferation. Hayashi et al. (1992) showed that cultured *Drosophila* embryo cells produce and are affected by mammalian nerve growth factor. Bone morphogenic protein (BMP) directs bone and epidermal cell development in vertebrates (Demetriou et al., 1996); the gene that elicits expression of BMP is homologous to the *Drosophila* gene decapentaplegic (Hemmati-Brivanlou and Melton, 1997), and is essential for *Drosophila* embryo development. Benzakour et al. (1990) reported a *Drosophila* KC cell line that produces a material similar to mammalian transforming growth factor (TGF) and a *Drosophila* gene expressing TGFβ has been isolated and sequenced (Padgett et al., 1987). Recently, Jiang and Edgar (2009) have shown that stem cells in larval *Drosophila* midgut are stimulated to proliferate via an epidermal growth factor receptor (EGFR) signaling pathway. In early larvae, the surrounding visceral muscles produce Vein, a weak EGFR ligand that stimulates a low level of stem cell proliferation. Later on in the larval stage, two other ligands are produced by the stem cells that provide signals that augment the first signal and lead to a higher level of proliferation. Interestingly, hematopoetic stem cells of vertebrates require developmental genes such as Notch, wingless, sonic hedgehog, and SMAD, which were originally identified in insects (Blank et al., 2008). Thus, it appears that the regulation of stem cell proliferation and metamorphosis may be a basic biological process controlled by similar regulatory peptides and processes. As I have drawn on information from the vertebrate stem cell literature for inspiration and ideas, I hope that this work on proliferation and differentiation of insect stem cells will be relevant to studies of stem cells of other animal species.

MIDGUT CELL CULTURE

Larval midgut from *Manduca sexta*, was first co-cultured in vitro with pupal fat body tissue in media containing low titers of the molting hormone, 20 hydroxyecdysone (20E) (Sadrud-Din et al., 1994). As the original epithelial explant degenerated during the first 7 days in vitro, undifferentiated cells, appearing as clear round or tadpole-like cells approximately 10 μm in diameter, containing large nuclei, were observed in the culture medium; other cells resembling differentiated goblet and columnar cells approximately 30 μm in length, similar to those observed in midgut epithelium in vivo, were observed in the cultures a few days later. As the number of round and tadpole-like cells decreased in the cultures, the number of differentiated goblet and columnar cell types increased. Approximately a week later, in the same cultures, the number of differentiated cells decreased as an increase in the number of undifferentiated cells was again observed (Fig. 1). These events occurred in regular cycles over a period of several months and suggested that the cultured stem cells gave rise to differentiated midgut cells. As the differentiated cells aged and began to die, new stem cells were produced (Sadrud-Din et al., 1994). Thus, cycles of Lepidopteran larval midgut

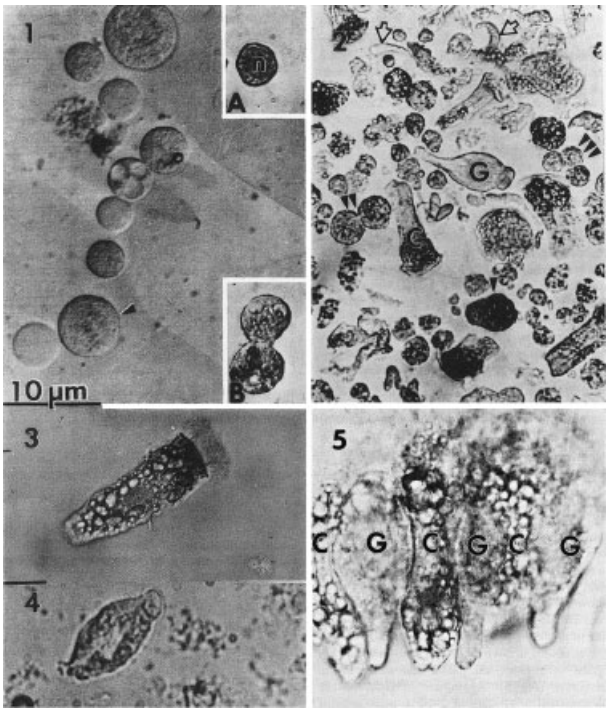

Figure 1. Cultured midgut cells from 4th instar larvae of *Manduca sexta*. **1:** Stem cells. **1A:** Granulated stem cell prior to division. **1B:** Dividing stem cells. N, nucleus. **2:** Overview of a mixed cell culture, showing stem, goblet (G) and columnar (C) cell types. Clear arrows indicate tadpole-like cells, triple solid arrowheads indicate dividing stem cells. **3:** Columnar cell. **4:** Goblet cell. **5:** Goblet (G) and Columnar cells (C) that have fused to form a monolayer of midgut epithelium. Bars = 10 µm.

development (Baldwin et al., 1996) appeared to be occurring in vitro. Similar events have been observed in vertebrate skin, gut, and blood wherein undifferentiated stem cells provide a supply of functioning differentiated cells to replace exhausted mature cells. These events are regulated by hormones and cocktails of peptidic, locally acting, growth factors (Burgess and Nicola, 1983; Rudland, 1993; Jones et al., 1995; Watt and Hogan, 2000).

Cultured midgut cells of *Manduca sexta, Heliothis virescens, Bombyx mori, Lymantria dispar*, and *Mamestra brassicae* were used in the work reviewed here.

HORMONAL FACTORS THAT INDUCE LEPIDOPTERAN MIDGUT STEM CELL MULTIPLICATION

Ecdysones

We found that low titers of 20E (1 ng/µl) were sufficient to maintain stem cell proliferation in cultured Lepidopteran midgut cells. However, Smagghe et al. (2005) showed that ecdysone (E) as well as 20E could serve as a midgut stem cell proliferation agent, although effectiveness was concentration-dependent (Smagghe et al., 2005). 20E was effective only in the absence of juvenile hormone (JH) in stimulating midgut stem cell proliferation in beetle midgut (*Tribolium castaneum*) in vitro (Parthasarathy

and Palli, 2008). Thus, E or 20E in the absence of JH appeared to provide the hormonal stimulus promoting continuous multiplication and differentiation of midgut stem cells in vitro.

α Arylphorin

Cell-free extracts of fat body (FBX) from pupae of *M. sexta* and *H. virescens* were prepared and assayed for effects on highly enriched preparations of stem cells obtained from cultured midgut (Loeb et al., 1999). As titers of FBX were increased, correspondingly more tritiated thymidine was incorporated by the stem cells and was converted to DNA, as evidenced by incorporation of the thymidine analog bromodeoxyuridine (BrdU) (Sadrud-Din et al., 1994; Loeb and Hakim, 1995a), strongly implying that FBX was indeed inducing stem cell multiplication (Fig. 2).

In order to detect the active ingredient in FBX, it was subjected to a series of High Pressure Liquid Chromatography (HPLC) and reverse phase HPLC separations. Fractions that induced significant increases in numbers of cultured stem cells were subsequently analyzed by Edman degradation (Blackburn et al., 2004). An active peptide of Mr 77 KDa was identified; its sequence aligned nicely with the known sequence of *Manduca sexta* α-arylphorin (Kramer et al., 1980; Blackburn et al., 2004). The arylphorins are high molecular weight (approximately 450,000 KDa) complexes composed of six subunits of α and β forms (Kramer et al., 1980). Although they are expressed primarily by the fat body of late instar larvae, they are released into the larval hemolymph, where they become the major protein component, and later accumulate in the fat body (Webb and Riddiford, 1988). Purified α-arylphorin (but not β-arylphorin) (Fig. 3), stimulated *H. virescens* midgut stem cells to multiply and continually form clusters of new cells after the 3rd to 4th day of continuous exposure, with as little as 125 ng/ml in the culture medium (Blackburn et al., 2004). Previously, it was postulated that the large titers of arylphorin synthesized by caterpillars about to enter metamorphosis served as storage protein, a source of amino acids for new tissues (Kramer et al., 1980; Webb and Riddiford, 1988). However, we showed that α-arylphorin could act as a powerful growth factor that stimulated stem cell mitosis, providing cells for new or enlarged tissues in subsequent stages of development.

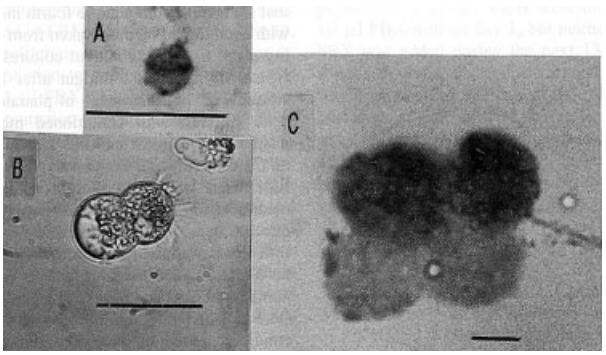

Figure 2. Dividing stem cells from pharate fourth instar *Manduca sexta* midgut. **A:** BrdU incorporation by chromosomes in a metaphase stem cell. **B:** Separating daughter cells, unstained (Nomarski optics). Note that the cell on the right has evidence of microvilli and may differentiate to a columnar cell, while the cell to the left appears smooth and may remain a stem cell. **C:** A cluster of separating daughter cell pairs, immunostained with BrdU. Note that one daughter cell of each pair has stained with BrdU while the other remained unstained. Bars = 5 μm.

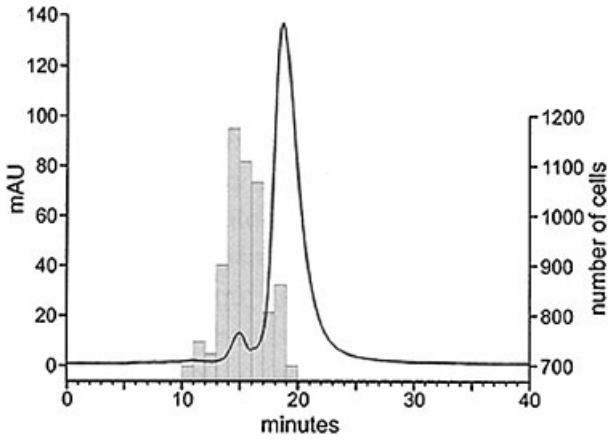

Figure 3. HPLC cation exchange fractionation of 250 µl of FBX (solid line). The superimposed histogram represents bioassays of each individual chromatogram fraction on stem cell numbers. Most activity was associated with the smaller peak in the absorption spectrum (mAU), which represents the alpha arylphorin fraction.

The mitogenic and growth-stimulating activity of arylphorin fed to larvae in vivo has been confirmed in a number of insect species (Hakim et al., 2001; Blackburn et al., 2004; Cermenati et al., 2007).

Bombyxin

Representatives of the insulin family of peptides, such as insulin-like growth factors I and II, have induced vertebrate (Furlanetto et al., 1997) as well as invertebrate cells (Hatt et al., 1997) to proliferate. Insulin promoted embryonic development (Nasonkin et al., 2002) and exerted control over body size in *Drosophila melanogaster* (Oldham et al., 2002). Bombyxin, isolated from *Bombyx mori* (Nagasawa et al., 1984), is a member of the insulin family of peptides. It regulates ovary maturation in *Drosophila* and wing disc growth in Lepidoptera (Nijhout and Grunert, 2002). When Goto et al. (2005) added Bombyxin to cultured midgut stem cells derived from *H. virescens* and *Mamestra brassicae* at concentrations as low as 10^{-12} M, stem cell number increased approximately 40% more than in control cultures. In contrast to α-arylphorin, Bombyxin acted almost immediately, and its effect declined to control levels by the 3rd day after application. Additional Bombyxin, administered to treated stem cells after 3 days, restored proliferation. Masumura et al. (2000) and Nijhout and Grunert (2002) have shown that the Bombyxin content of brains of starved larvae increases, and then decreases when larvae are fed again. Thus, Bombyxin may quickly regulate the supply of midgut cells needed for digestion in response to the food supply in vivo. Bombyxin appears to be a quick-acting but temporary midgut cell growth-proliferation factor while α-arylphorin may be a slower-acting but longer-term growth-proliferation factor for Lepidopteran midgut stem cells.

MIDGUT DIFFERENTIATION FACTORS

Cell-free conditioned medium, prepared from mixed midgut epithelium cultures, promoted midgut stem cell differentiation to columnar and goblet cells in vitro

(Loeb and Hakim, 1995a, 1996). Differentiated cells characteristic of 4th instar midgut epithelium, bearing true goblet and columnar cells, resulted from incubation of stem cells in medium taken from 4th instar midgut cultures. However, medium taken from midgut cultures of wandering 5th instar larval midgut cultures and applied to the same colony of stem cells induced differentiation of typical wandering 5th instar midgut cells; short granular columnar cells and incomplete goblet cells were observed. These data suggested that more than one growth factor controlled specific differentiation pathways (Loeb and Hakim, 1995b, 1996).

All of the active factors in conditioned media were destroyed by incubation with peptidase, were heat stable at 100°C, and appeared to be small peptides of less than 10 KDa.

Midgut Differentiating Factors (MDFs) 1 and 2: Separation and Sequencing

Heated cell-free conditioned medium from cultures of 4th instar *H. virescens* midgut was subjected to a series of HPLC and Edman degradation procedures. The most active material turned out to be a 30-amino acid peptide with the sequence HVGKTPIVGQPSIPGGPVRLCPGRIRYFKI (Mr 3,244). A somewhat smaller active peptide of 26 amino acids, HVGKTPIVGQPSIPGGPVRLCPGRIR (Mr 2,689), which we called Midgut Differentiation factor 1 (MDF1), was commercially synthesized (Quality Controlled Biochemicals Inc., Boston, MA). It promoted maximum differentiation of *H. virescens* larval-type stem cells at concentrations of 10^{-14} to 10^{-8} M within 7 days (Loeb et al., 1999).

MDF1 was not specific to insect cells. An Internet NCBI Blast search of its sequence showed it to be identical to the carboxy terminal sequence of bovine fetuin, spanning residues 330 to 359. Fetuin is a high-molecular-weight glycoprotein constituent of the fetal calf serum used to culture midgut cells. In vertebrate embryos, fetuin increases proliferation of bone marrow cells, and can act in concert with, or antagonistically to, the transforming growth factor (TGFβ) family of growth factors and to bone morphogenic protein (BMP), a peptide that directs bone as well as epidermal cell development in vertebrates (Demetriou et al., 1996). Surprisingly, intact bovine fetuin did not induce stem cell differentiation, although its hydrolysate was quite potent. Mixed midgut cell cultures and intact midgut epithelium contain differentiated columnar cells that are rich in trypsins, chymotrypsins, elastases, carboxypeptidases, and dipeptidases (Terra et al., 1996). Many of these enzymes are located on columnar cell microvilli, which may break off into the culture medium just as they are normally released into the gut lumen in vivo (Billingsley and Lehane, 1996), and may provide the enzymes necessary to hydrolyze fetuin from the culture medium to MDF1 in vitro. Cultures enriched in stem cells contain few columnar cells and probably lack enough of the proteases needed to liberate MDFs from fetuin into the medium. The cellular reaction must be specific for the portion of the fetuin molecule peptide we called MDF1, since the activity in a separately prepared fetuin hydrolysate was confined to a small peptide, identified by methods similar to those already described, as HRAHY, a sequence *not* found in MDF 1; it induced 70% differentiation of stem cells in 8 days in culture and was active from 10^{-15} to 10^{-5} M. We called this second peptide MDF 2. Although MDF1 promoted the development of typical 4th instar *H. virescens* columnar and goblet cells from isolated stem cells, MDF 2 caused approximately 58% of about 3,000 newly differentiated cells to become cuboidal in shape, each with a central nucleus and a microvillar fringe on one side,

a phenotype observed in cultures prepared from wandering, prepupal, and adult midguts (Loeb et al., 2003).

MDFs 3 and 4 from Hemolymph of Lymantria dispar

Since *Lymantria dispar* pupae are large, were available, and provided relatively large quantities of hemolymph, we used them as a hemolymph source. Untreated hemolymph of pupal *Lymantria dispar* had little effect on stem cells. However, when it was heated to 100°C and then hydrolyzed with chymotrypsin, it did indeed induce differentiation of enriched *H. virescens* stem cells. Chymotryptic digests of the hemolymph were subjected to a series of RP-HPLC column separations and Edman degradation. Two active fractions with sequences EEVVKNAIA and ITPTSSLAT, quite different from those of MDFs 1 and 2, were designated MDF3 and MDF4. These peptides were also commercially synthesized. Each was very active in inducing 4th instar-like differentiation of *H. virescens* midgut stem cells. Peak activity was detected at 10^{-6} M (EEVVKNAIA) AND 10^{-8} M (ITPTSSLAT), respectively, well within the physiological range for bioactivity (Loeb and Jaffe, 2002).

Combining MDFs 1 and 2 provided an increase in differentiation activity of about 60% compared to 45% alone after 7 days of incubation. But combining MDFs 1 and 3 provided no more stimulus than one of the MDFs alone over a range of concentrations; 2 plus 3 or 2 plus 4 at 10^{-8} to 10^{-9} M eliminated effectiveness to control levels and almost completely inhibited differentiation at 10^{-10} M.

This work supports the hypothesis that cocktails of interactive growth factors may fine-tune developmental fates of midgut stem cells.

MDF-1 in Midgut Epithelium In Vivo

Antibody to synthetic MDF-1 was commercially prepared (Cocalico Biologicals, Inc., Reamstown, PA). Immunoblots indicated strong affinity for anti-MDF1 and fetuin, and weak affinity for extracts of the food used to grow the larvae. Cultured midgut cells and midguts dissected from 4th instar *H. virescens* larvae were fixed and prepared for histological examination. Slides were exposed to 1% bovine serum albumin to block non-specific-binding sites and incubated with anti-MDF-1. Color was developed using Fast^{TM} BCIP/NBT (5-bromo-4 chloro-3-indoryl phosphate/nitro blue) (Sigma, St. Louis, MO). Only columnar cells stained intensely with anti-MDF 1. Goblet, stem, endocrine, and differentiating cells as well as muscle, blood cells, or tracheoles in the sections rarely if ever were weakly positive to anti-MDF 1 antibody. Most intensely staining were columnar cells from pre-molting, slipped-head larvae (Goto et al., 2001) at the time that stem cells are present in greatest number (Baldwin and Hakim, 1996). The basal and basolateral cytoplasm of these columnar cells was strongly immuno-positve, while apical cytoplasm adjacent to the microvilli remained unstained or lightly stained. The microvilli were darkly stained (Fig. 4), suggesting a role in either capturing fetuin-like material, breaking it down to MDF 1-like segments, or sequestering MDF 1 before passing it into the columnar cells. Sections exposed to antibody preabsorbed with anti-MDF1 did not stain, verifying that the reaction to anti-MDF1 was specific (Goto et al., 2001). Sections of larval midgut from larvae never exposed to laboratory fetuin, stained intensely, suggesting that a fetuin-like antigenic molecule and/or MDF1 was present in intact animals. Amino acids, sugars, and proteins ingested by columnar cells at the midgut lumen are moved through the cells and delivered to the hemolymph by actively crossing the basal membrane (Sacchi and

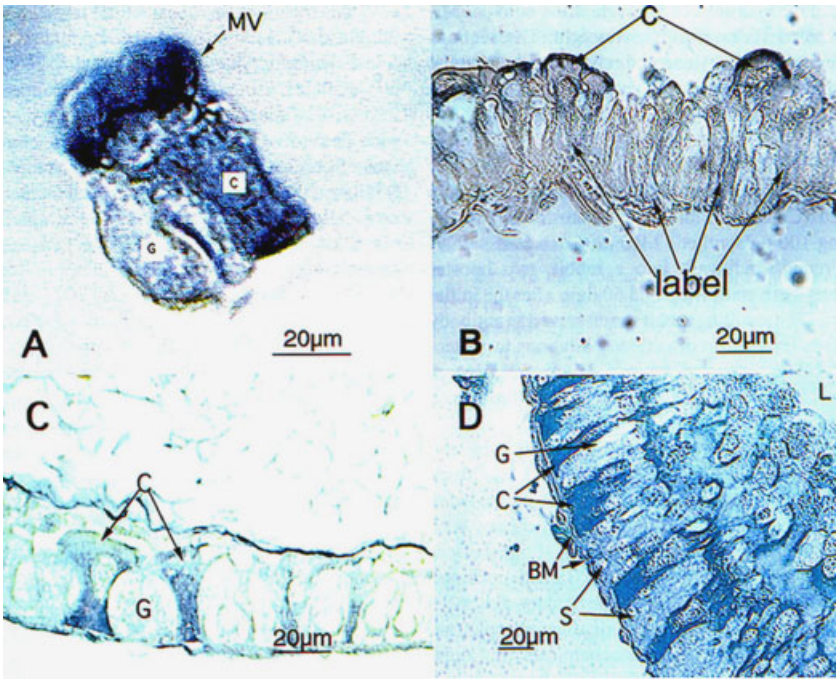

Figure 4. Midgut cells of *Heliothis virescens* were exposed to rabbit antibody to MDF 1 and visualized with anti-rabbit IgG conjugated to alkaline phosphatase; color was developed using FAST^{TM} BCIP/NBT (Sigma). **A:** A row of cultured midgut cells. Note staining of microvilli (MV) and base of columnar cell (right). **B:** Cross-section of midgut epithelium excised from a newly molted 4th instar larva. Microvilli and bases of columnar cells are lightly labeled. **C:** Section of midgut of 4th instar feeding larva. Intense immunostaining can be seen in basal areas of columnar cells (C) cells but not in goblet cells (G). **D:** Midgut from pre-molt larva. Basement membrane (BM) marks the basal end of the epithelium. Mid and basal columnar cell cytoplasm (C) stains intensely but bunches of stem cells (S) do not stain. Bars = 20 µm.

Wolfersberger, 1966; Cermenati et al., 2007), suggesting a method for moving fetuin or fetuin-like compounds and/or their hydrolysates into and through columnar cells. It may also be possible that MDF 1 is synthesized within columnar cells de novo.

MDFs 2, 3, and 4 in Midgut Epithelium In Vivo

Slides of cultured midgut cells and midguts from 4th instar larvae of *H. virescens* were incubated with antibodies prepared from MDFs 1, 2, 3, and 4 (Cocalico Biologicals, Inc.), alone or in combination. The microvilli of cultured columnar cells were strongly immunopositive to antibody to MDF-2 as well as MDF-1. However, all of the cytoplasm of columnar cells was immunopositive to anti-MDF-2 instead of just the basal areas positive to anti-MDF-1. More significantly, anti-MDF-2-stained droplets were observed in the midgut lumen, perhaps released by columnar cells, suggesting a digestive function as well as its effect on stem cell differentiation.

In contrast, anti-MDF-3 stained the central areas of cultured midgut columnar cells, although it was found at the bases of columnar cells in whole midgut.

Secretory cells were the only cell type that stained with anti-MDF-4; streams of MFD-4-positive material were observed extending from the secretory cells near the lumen side of the epithelium, and as a layer on the hemolymph-facing portion of the

epithelium, suggesting an endocrine or paracrine function for this or a similar immunoreactive peptide (Fig. 5) (Loeb et al., 2004). Antibody to MDF4 also strongly stained secretory cells of *Drosophila* midgut, indicating its presence in a non-Lepidopteran (Takeda, personal communication, 2008).

Effects of Non-Insect Growth Factors and Neuropeptides in Regulating Cell Fates

Over 60% of freshly isolated larval midgut stem cells from *H. virescens* (approximately 10 μm in diameter) exposed to MDF1 differentiated to larval forms of columnar or goblet cells approximately 30 μm long within 6–8 days (Loeb et al., 1999; Loeb and Jaffe, 2002). In contrast, after 15 days of exposure to platelet-derived growth factor (PDGF), approximately 11% of the cells differentiated to oval cells, approximately

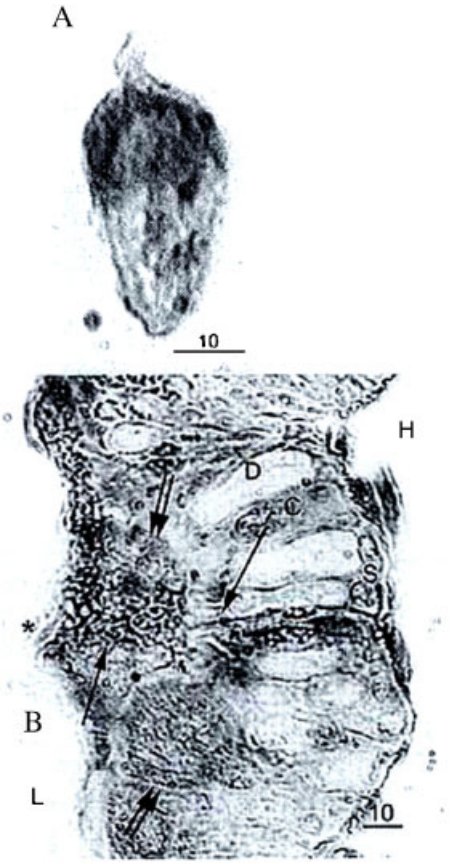

Figure 5. Midgut cells immunostained with antibody to MDF 4. **A:** Endocrine cell from *H. virescens* midgut culture incubated with MDF 4, with color developed as described in the text. Secretion is stained within the cell. Bar = 10 μm. **B:** Section of midgut from a late last instar larva of *H. virescens* incubated with *MDF 4*, with color developed as described in the text. Single arrows point to putative streams of stained secretion apparently emerging from a large secretory cell (double arrows) in the center of the figure. Secretion appears to be flowing to the hemolymph (H) side of the midgut epithelium and stained material is also visible on the lumen (L) side (*). Another immunopositive secretory cell appears at the bottom of the figure (lower double arrows), releasing stainable material to the lumen side, but little to the hemolymph side of the epidermis. **C**: Mature columnar cell. **D**: Differentiating columnar cell. S, Stem cells. Bar = 10 μm.

60 μm long, each with a prominent vacuole and no visible microvilli. Epidermal Growth Factor (EGF) and all-trans-retinoic acid (RA) induced stem cells to multiply approximately 1.5-fold more than untreated controls after 48 hours. By 7 days in culture, daughter cells had enlarged to approximately 20 μm in diameter. Twenty-five percent of the large daughters further flattened to form irregular shapes with central nuclei, approximately 60 μm in diameter that resembled vertebrate squamous epithelial cells (Fig. 6). In some of the wells containing RA, we occasionally observed insect scale-like cells as well as the squamous-like cells seen with EGF (Loeb et al., 2003). These cell types were not observed in lepidopteran larval midgut in vivo, or in untreated midgut cultures in vitro (Sadrud-Din et al., 1996; Loeb and Hakim, 1996). Thus, these non-insect growth factors caused midgut stem cells to assume non-midgut fates, indicating multipotency of the stem cells in the presence of "foreign" peptides.

Jiang and Edgar (2009) showed that stem cells in larval *Drosophila* midgut can be stimulated to proliferate by an EGFR signaling pathway. Therefore, EGF may be effective in regulating midgut stem cell fate in more than one insect group.

Stem cells incubated with adipokinetic hormone, FMRFamide, insulin, proctolin, allatostatins, or with mammalian factors melatonin, nerve growth factor, transforming growth factor (TGFβ), or luteotrophic hormone at physiological concentrations appeared visibly unaffected by these agents (Loeb et al., 2003).

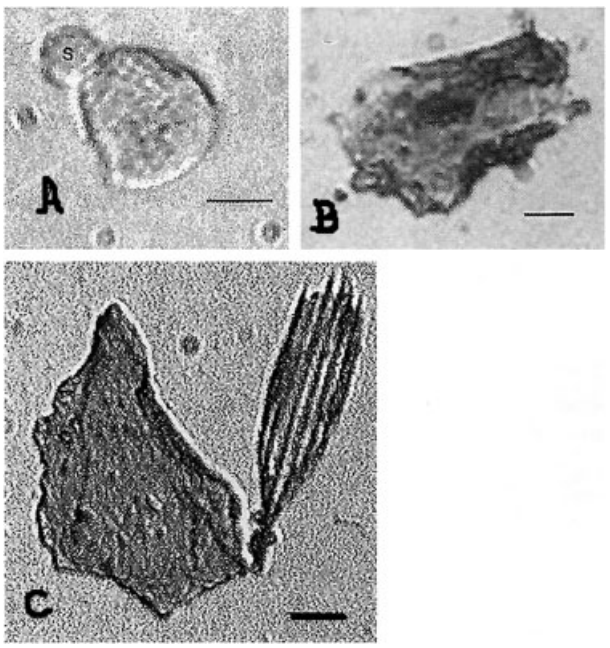

Figure 6. Effects of mammalian growth factors on cultured stem cells of *Heliothis virescens*. **A:** Dividing stem cell exposed to retinoic acid (RA)(10^{-6} M) for 10 days. One daughter cell is normal size while the other is differentiating to something larger. S, stem cell. Bar = 20 μm. **B:** Squamous-like cell derived from stem cells exposed to epidermal growth factor (10^{-6} M) for 14 days. Bar = 10 μm. **C:** Squamous-like cell (left) and scale cell (right) from stem cells exposed to RA (10^{-6} M) for 14 days. Bar = 10 μm.

Calcium Ion (Ca^{2+}) Effects

Calcium ion is a first and second messenger that drives numerous cellular processes (Berridge, 1983). There is a tremendous efflux of Ca^{2+} from the midgut to the hemolymph of larval Lepidoptera (Wood and Harvey, 1976) that may be facilitated by an ATP-dependent Ca^{2+} pump that we identified in plasma membranes isolated from *L. dispar* midgut (Sheppard and Loeb, 1992). Alteration of the calcium titer of the culture medium surrounding midgut stem cells of *H. virescens* had a profound effect on stem cell fate (Loeb, 2005). Decreasing Ca^{2+}_{out} by use of the Ca^{2+} chelating agent ethylene glycol-bis (2-amino ethyl ether)-N, N, N′N′-tetraacetic acid (EGTA) promoted dose-dependent stem cell multiplication up to 1.5-fold and differentiation to larval forms of midgut epithelium in vitro, similar to that induced by the MDFs (Loeb et al., 1999; Loeb and Jaffe, 2002). In contrast, increasing the concentration of Ca^{2+}_{out} by adding Ca^{2+} to the culture medium to a concentration equal to or more than 55 mM, or increasing Ca^{2+} entry into stem cells by use of the calcium ionophore A23187 (Rosenberger and Triggle, 1978), stimulated cultured larval midgut stem cells to produce scale-like, squamous-like, and vacuolated cells in a dose-dependent fashion, similar to effects of mammalian growth factors PDGF, RA, and EGF. Thus, changes in Ca^{2+} concentration inside and outside midgut stem cells may act as second messengers for peptidic signals that change midgut cell fates, some of which may not be part of the normal midgut developmental pathways.

SUMMARY

In vivo, pre-molt larval lepidopteran midgut stem cells multiply rapidly just before they differentiate to mature forms of midgut epithelial cells (Baldwin et al., 1996). In vitro, stem cells multiply in the presence of low titers of steroid hormones E (Smagghe et al., 2005) or 20E (Sadrud-Din et al., 1994) in the absence of juvenile hormone (Parthasarathy and Palli, 2008), mimicking the hormonal balance that exists prior to a molt (Riddiford, 1994). We have shown that at least two peptidic factors control stem cell multiplication: the fast-acting and quickly exhausted brain peptide bombyxin (Goto et al., 2005) and the slower-acting, longer-lasting blood and fat body peptide, α-arylphorin (Blackburn et al., 2004). However, it is still unknown how these factors enter midgut cells, how they are processed, or how they incite stem cells to divide. The predominant mature cells observed in midgut epithelium change as the role of the midgut changes from an organ of digestion and transport of nutrients in mostly leaf-feeding larvae to one of storage and metabolic support in non-feeding wandering, pre-pupal and pupal stages and to those in adult moths that sip nectar (Waku and Sumimoto, 1971; Baldwin et al., 1996). Specific stem cell fates can be elicited by cell-free media in which a particular developmental stage of the midgut epithelium had grown, indicating that different growth factors were released into the media by the different mature cells in the cultures. We have described and characterized a few growth factors, the MDFs (Loeb et al., 1999, 2003; Loeb and Jaffe, 2002), and have elicited differentiation to other cell forms by commercially available vertebrate growth factors (Loeb et al., 2003), emphasizing the complexity of regulation of midgut stem cell differentiation and the plasticity of lepidopteran midgut stem cells. Vertebrate stem cells multiply and function under the control of a bevy of locally interacting peptidic growth factors (Watt and Hogan, 2000), and may also occur as developmental

fates of lepidopteran stem cells are regulated. We have located MDFs immunologically within particular areas of mature columnar and endocrine midgut cells (Goto et al., 2001; Loeb et al., 2004), but have yet to show whether MDFs are synthesized within these cells or are only sequestered there, how they are released, how they exert their control over differentiation or digestion, or whether they have roles in regulating other functions of the midgut or tissues located elsewhere. It is particularly puzzling to know that columnar cells stain with antibodies to MDFs, and yet differentiating stem cells stain poorly, if at all, with those antibodies. We have some evidence, also obtained via histochemistry, that some of the MDFs are released to the gut lumen, perhaps to influence digestion, while those from the secretory cells reach the gut lumen as well as the hemolymph, where they may have a paracrine function in addition to metamorphic effects. We do not know the mechanisms by which the factors produce their results, although Ca^{2+} experiments (Loeb, 2005) suggest second-messenger mediation. The experiments described in this missive have just barely raised the curtain on the determinants of midgut stem cell fate in Lepidoptera. It is hoped that current work on regulating vertebrate stem cell fates will provide clues and incentives to continue these studies in insects.

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